An Individual-based Model for Simulating Antibiotic Resistance Spread in Bacterial Flocs in Wastewater Treatment Plants

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Abstract

Wastewater treatment plants (WWTPs) receive wastewater that carries a variety of pollutants, including antibiotics and antibiotic-resistant bacteria. The potential for horizontal gene transfer of resistance through conjugation – direct cell-to-cell transfer of genes carried on a plasmid – is high in WWTPs because of high cell density and residence time in bacterial flocs. To better understand how resistance spreads by growth and conjugation in such flocs, we propose an individual-based model with a solver algorithm for dynamic simulation. Our model includes only the most relevant bacteria properties and functions such as movement, growth, division, gene transfer, and death. Simulation of our model suggests that resistance can increase by conjugation at the early growth stages of a floc and that the overall rate of gene transfer depends on floc size. Results indicate that our simple model can be a useful tool for examining how gene exchange and heterogeneity contribute to the spread of antibiotic resistance in bacterial flocs.

Keywords: antibiotic resistance, wastewater treatment, individual-based model, simulation

1 Introduction

Antibiotics are important pharmaceuticals for the treatment of infectious bacterial diseases (Hellweger *et al.*, 2011; Sabri *et al.*, 2020). Overuse and misuse of antibiotics have led to the increased development of antibiotic resistance (AR) (Duarte *et al.*, 2019). AR was estimated to be responsible for at least 700 thousand deaths in 2014 and is estimated to claim 10 million lives yearly by 2050, more than other major diseases such as diabetes and cancer (O'Neil, 2016).

Biological wastewater treatment plants (WWTPs) are environments with high potential importance for the spread of AR (Uluseker *et al.*, 2021). WWTPs contain rich microbial populations with very high cell densities (around 10^8 – 10^{10} cells per mL) supported by high nutrient availability (Jenkins and Wanner, 2014). Raw sewage originates from various sources and can contain large numbers of resistant bacteria (Hassoun-Kheir *et al.*, 2020). Studies have shown that resistance levels stay high throughout WWTPs (Amarasiri *et al.*, 2020; Gao *et al.*, 2012). Most resistant bacteria are fortunately removed together with other microorganisms during the final settling and sedimentation stages; the concentration of resistant bacteria in the sludge, however, can be as high as in the inlet raw sewage (Gao *et al.*, 2012). Resistant bacteria can proliferate in WWTPs, and they can spread their resistance genes to nonresistant bacteria through horizontal gene transfer (HGT). This is worrisome as resistance can spread from pathological bacteria that arrive with the wastewater to aquatic and soil bacteria that are well adapted to both the WWTP environment and to river and soil environments that receive WWTP effluents and biosolids.

Bacterial conjugation is a natural process of plasmid transfer between bacteria. A bacterium that contains a plasmid with one or several resistance genes can by conjugation transfer a copy of this plasmid to other bacteria, but only to bacteria that are compatible with the plasmid and the biological conjugation process (Koraimann *et al.*, 2004). Plasmids that can be shared through conjugation are called conjugative plasmids, and bacteria that can receive and transfer a conjugative plasmid are said to be competent. Such plasmids can carry antibiotic resistance genes (ARGs) and replicate in a wide range of host bacteria (Krone *et al.*, 2007).

In order to address the effect of bacterial growth and conjugation on the spread of AR in WWTP bacterial aggregates, we have designed an individual-based model (IbM) where each bacterium cell is a single and discrete entity that has its own internal state and that interacts only with its closest neighbours. Our model captures local heterogeneity and local interactions and can be used to simulate how resistance genes are transferred by conjugation within a bacterial floc. The IbM is constructed as a minimum model but includes key processes to capture the spread of ARGs.

2 Modelling

Individual bacteria are in the model placed in a *bacteria position grid* where each point in the grid corresponds to a position in the environment. There is also a substrate grid that is used to keep track of the concentration of the growth-limiting substrate, S, at each location and to model how substrate diffuses into the bacterial floc. Individual bacteria are described by their main processes: substrate uptake and cell growth,

reproduction, and cell death. Individual bacteria can interact with neighbours by conjugation of resistance plasmid and displacement (shoving), which happens as bacteria grows. The model is developed with a minimum of complexity to be suitable for simulating the spread of AR in a growing floc. It is implemented according to the Overview, Design and Details (ODD) standard protocol proposed by Grimm *et al.* (2006) and implemented in MATLAB.

Each bacterium has the following state variables: - position *P*, the bacterium's current position in the environment; - cell size *X*, the current dry mass of the bacterium; - resistance *R*, indicating whether the bacterium is resistant or not; - conjugation compatibility *C*, indicating whether the bacterium has the molecular machinery for horizontal gene exchange; - and remaining recovery time *T*, indicating the remaining recovery time after a conjugation event, i.e., the time before a donor or receiving bacterium again is capable of exchanging genetic material.

Our algorithm for simulation of bacterial growth and resistance gene transfer starts with individual bacteria being placed randomly in a defined region within the environment. This region represents the bacterial floc. A selected number of the cells are initialized, some with and some without resistance. Resistant bacteria are defined as carriers of a resistance plasmid. The substrate grid is also initialized. The algorithm then starts simulating the temporal evolution of the floc by an outer loop where shoving of overlapping bacteria and substrate diffusion occurs. For each temporal iteration, an inner loop tracks through all the individual cells. The algorithm is summarized in the flow chart in Figure 1 and model parameters are shown in Table 1.

The algorithm is explained in detail in the following:

Cell movement: Bacteria in the floc are considered non-motile, i.e., they do not move actively. Displacement of bacteria from shoving due to growth and division is implemented by a shoving mechanism based on Kreft *et al.* (2001). The bacteria are considered as hard spheres in a 2-dimensional plane. The radius of a bacterium is calculated as:

$$r = \sqrt[3]{\frac{3X}{4\pi\rho}} \tag{1}$$

where X is the cell size (in dry mass) and ρ is the density of the bacterium.

The overlap distances d_i^j from bacterium i to bacterium j *is* then calculated by using the formula:

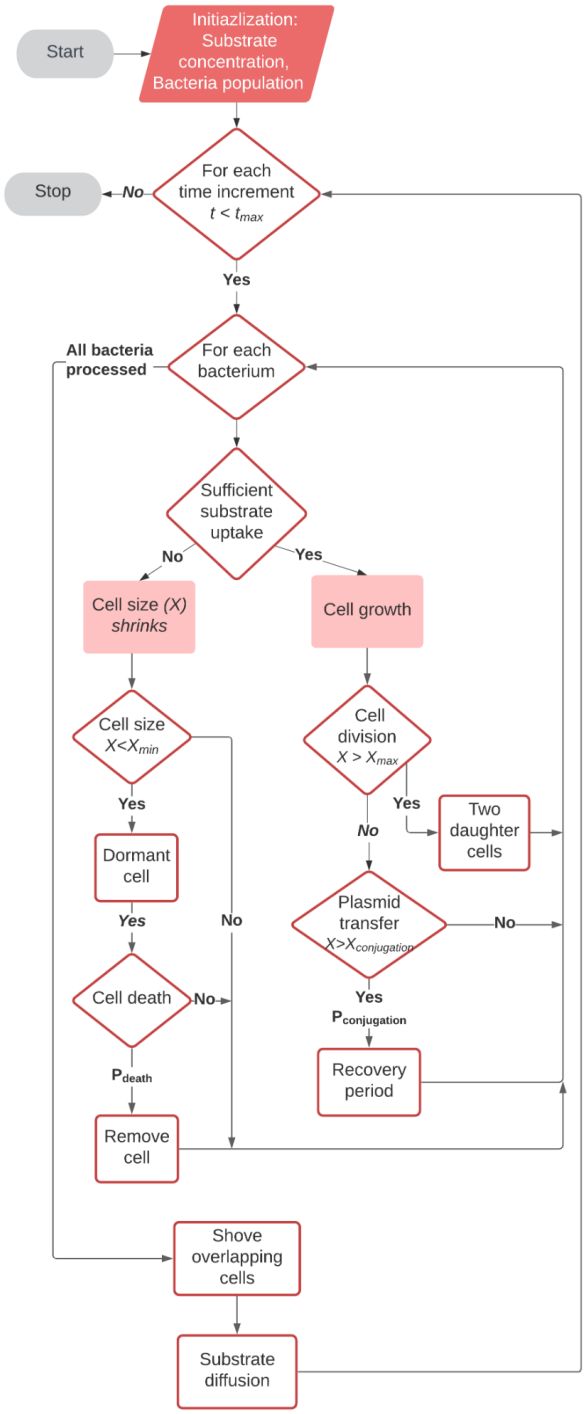

Figure 1. Flow chart of the simulation algorithm. Division, gene exchange, shoving and death are probabilistic events that depend on the properties of the individual cell and its neighbours. See the main text for a detailed explanation of the different steps.

$$d_i^j = kr_i + r_j - \left|\left|P_i - P_j\right|\right|_2 \tag{2}$$

where P_i and P_j are the position coordinates of bacterium i and j respectively, and k is a constant that accounts for maximal bacterial density or as Kreft *et al.*

(2001) states, the minimum spacing. The shove vector for bacterium i is then calculated as:

$$\Delta P_i = \sum_{j \in G_i} d_i^j \frac{P_i - P_j}{\left|\left|P_i - P_j\right|\right|_2} \quad (3)$$

where G_i is the set of bacteria that overlap with bacterium *i*, i.e., all bacterium *j* for which the overlap distance d_i^j is positive.

The maximum distance between bacteria that can have a positive overlap distance is $(k+1)r_{max}$. Since only positive overlap distance is needed in the shoving algorithm, all the potential members of G_i is found by only checking bacteria localized at grid points in the bacteria location grid that have a distance less than $\frac{(k+1)r_{max}}{2}$ away from the grid point containing bacterium *i*. The grid resolution is specified so that the 8 connected neighbourhood grid points contain all potential members of G_i.

Substrate diffusion: The substrate grid is initialized with a given substrate concentration at each point. The substrate is used during bacteria growth and a part of the bacteria dry mass is returned as a substrate when bacteria die. Therefore, the substrate concentration (in a location) is not constant, and the substrate will diffuse towards lower concentrations. A simple diffusion algorithm based on Kreft *et al.* (1998) is used where a 2-dimensional filter *DF* is used to calculate the transfer of the substrate from each point in the grid to the 8 adjacent points.

$$DF = \begin{bmatrix} 1 & 4 & 1 \\ 4 & -20 & 4 \\ 1 & 4 & 1 \end{bmatrix} \quad (4)$$

The concentration at the border of the grid is kept constant and the substrate grid is updated according to:

$$S_{grid,t+1} = S_{grid,t} + d_k(DF * S_{grid,t}) \quad (5)$$

where d_k is a diffusion constant that accounts for the diffusion coefficient and the length of each time step and $*$ is the convolution operator.

Bacteria functions and interactions: At every time step each cell performs the following:

1. *Substrate uptake:* The bacterium takes up nutrients from the substrate grid corresponding to the bacterium's current position. The concentration in the substrate grid is immediately updated. The substrate uptake rate is determined by a saturable function that depends on substrate concentration and cell size according to Monod kinetics:

$$v = V_{max} X \frac{S}{K_m + S} \quad (6)$$

where V_{max} is the maximum uptake rate per unit of dry mass and K_m is the half-saturation constant.

2. *Growth and maintenance:* Nutrients taken up from the environment are used for cell growth with efficiency according to a yield constant *Y*. The maintenance rate, i.e., how much substrate is used for non-growth metabolism, is modelled to be linearly dependent on cell size. The total growth rate is given by:

$$\Delta X = Yv - k_{maintenance} X \quad (7)$$

Resistant bacteria are modelled to have a 1% lower growth yield than non-resistant bacteria due to the metabolic burden of producing resistance enabling proteins (Gregory *et al.*, 2008). The effect of the metabolic burden is that it reduces the growth rate and increases the generation time. The cell shrinks if the available nutrients are insufficient for growth.

3. *Dormancy and Death:* If the cell size is below X_{min} the cell is starved and becomes dormant – a state where growth and maintenance stop. At each time step, a dormant bacterium may die with probability P_{death}, in the algorithm determined by the generation of a random number.

4. *Division:* If the cell size is above the normal size of a full-grown cell, X_{max}, the cell divides. A neighbourhood position is randomly selected and the cell divides by displacing occupants of the neighbouring position. The cell divides into two daughter cells where both cells get 40% of the size of the mother cell, and the remaining 20% of the mother cell is divided randomly between the two daughter cells. This unequal size helps to disrupt synchronous growth in the model. If the mother cell is resistant there is a probability P_{loss} that resistance is not transferred to the daughter. Resistant bacteria are competent for conjugation and there is a chance $P_{competent}$ that nonresistant bacteria become competent for conjugation during cell division or when they are added to the environment.

5. *Gene exchange:* If the cell size reaches $X_{conjugation}$, which is 80% of full-grown size X_{max}, and the cell is resistant it might spread its resistance to a competent nonresistant cell in its neighbourhood (Park *et al.*, 2018). Plasmid transfer has been estimated to happen on average at a rate of 10^{-3} per individual cell-to-cell interaction (Gregory *et al.*, 2008). Each time step, each potential donor cell checks whether neighbourhood cells are competent and nonresistant. If they are, conjugation occurs with probability $P_{conjugation}$. Plasmid transfer to the receiving nonresistant bacterium happens instantaneously. Under the condition that conjugation occurs, there is a recovery time (*T*) before the donor and

recipient cell can transfer a new plasmid (Gregory *et al.*, 2008).

Table 1. Simulation parameters. References: [a] (Kreft *et al.*, 1998), [b] (Kreft *et al.*, 2001), [c] (Gregory *et al.*, 2008), [d] (Park *et al.*, 2018). The values for unreferenced parameters are arbitrarily decided under the condition that they are plausible compared to the value of other parameters and that they give a reasonable system response. All parameters are given in arbitrary units.

Parameter description	***Parameter Value***
Grid size	500x500
X_{min}, minimum cell size[a]	0.1
X_{max}, maximum cell size[a]	0.5
k, minimum spacing[b]	1.3
K_m, half-saturation constant	0.01
$k_{maintenance}$, maintenance rate	0.002
Y, yield constant[a]	0.4
Cost of resistance on growth[c]	1%
Plasmid transfer rate[c]	10^{-3} per interaction
$X_{conjugation}$, cell size for conjugation[d]	80% of X_{max}

3 Results

We will here present some results from using our model to simulate how resistance spreads through conjugation and growth in a growing bacterial floc. Three different simulations have been implemented to illustrate how the two factors recovery time and probability for competence affect the spread of resistance.

The first simulation describes a small floc that starts with equal amounts of ten resistant and ten competent nonresistant bacteria with parameters from Table 1 and a modest recovery time of 200 time steps. The probability for nonresistant bacteria to be competent is set to 20%.

The second simulation is similar to the first one except for a longer recovery time of 300 time steps with a similar 20% probability for competence. This simulation is set up to illustrate the importance of recovery time on the spread of resistance in the floc.

A final simulation is made to understand the specific effect of the probability for competence. The probability for nonresistant bacteria to be competent is set to 10% while keeping the recovery time similar to simulation 1, as 200 time steps.

Note that the system parameters are set in arbitrary units. Our intention is to illustrate the function and qualitative behaviour of the model and not necessarily to show results that have a one-to-one relationship in specific biophysical units. Quantifying the model parameters with proper biophysical units would require experimental data and we will leave this for future development.

3.1 Gene exchange and resistance

For the first simulation, the floc is set up to initially contain ten resistant and ten nonresistant competent bacteria. The probability for a bacterium being competent, $P_{competent}$, is set to 0.2 and the recovery time, T, is set to 200 time steps, which is higher than the typical division time. Figure 2 provides snapshots of floc development for resistant (red) and nonresistant bacteria (blue) and transconjugant bacteria (green) during simulation. In the early stages of floc development, conjugation occurs in most of the floc. At t=500 most of the floc has become resistant as shown in the population plot in the bottom right of Figure 2. Transconjugants, bacteria that have received a plasmid are marked green to express the frequency of conjugation in the floc. At the later stages of floc development (t > 1000) conjugation is limited and happens mainly in the intersection of resistant and nonresistant bacteria at the edge of the floc. This is because most of the bacteria inside the floc are already resistant and because nutrient limitations, illustrated by the background colour that changes from white to grey and black as the substrate concentration reduces, cause bacteria in the deeper part of the floc to become dormant. At t=1000 regions of nonresistant bacteria surrounded by resistant bacteria have emerged, primarily due to shoving. Moreover, transconjugant bacteria occur on the border of the protrusions. The increase in resistance is after t=1000 mostly from growth. At t=4000, conjugation stops playing any significant part since resistant and nonresistant bacteria form and grow on different protrusions.

The population plot to the bottom right of Figure 2 summarises the resistant and nonresistant bacteria population during the simulation. It is apparent that since the very beginning of the simulation, resistant bacteria population in the floc is higher than nonresistant bacteria. In this case, it is seen that the population of resistant bacteria increase even though the resistant cells have a higher metabolic burden and a reduced growth rate compared to nonresistant cells.

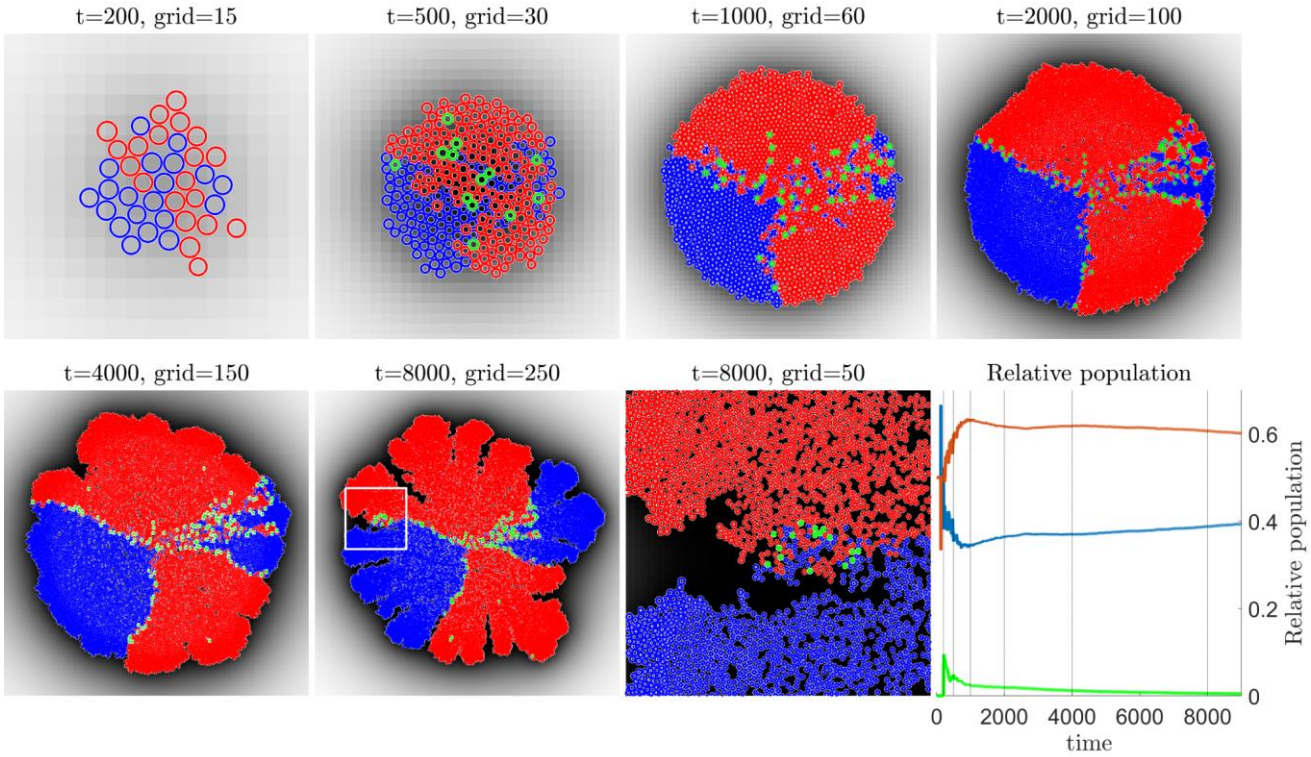

Figure 2. Population plot (lower right) and snapshots of floc development (other panels) from simulation 1. Timestep and size of the viewed grid are shown above the panels. Resistant (red), nonresistant bacteria (blue) and transconjugants (green). Substrate concentration is constant at the border of the nutrient grid and diffuses toward lower concentration, i.e., into the floc (grayscale, white is high concentration). Initially, at t=0 (not shown), ten resistant (red) and ten nonresistant bacteria are initialized in the middle of the environment. The probability of nonresistant bacteria being compatible is 20% while all resistant bacteria are competent. Conjugation recovery time is 200 time steps. At each time step, t, the simulation algorithm is run once. The rightmost snapshot of timestep 8000 (lower middle) shows a further zoomed in image of the floc shown in the snapshot to its left and illustrates how the resistant and nonresistant bacteria grow on separate protrusions.

3.2 Effect of longer recovery time

The second simulation is conducted to examine the effect of the recovery time on the spread of resistance. The recovery time, *T*, is increased to 300 time steps. Initial conditions and probability for competence are identical to the previous simulation, and ten resistant and ten nonresistant are placed in the middle of the environment at t=0. Figure 3 displays the snapshot results and the change in the bacteria population during the simulation. The results indicate that the relative bacteria populations are highly affected by the recovery time, *T*. As expected, increasing the recovery time decreases the resistant bacteria population in the floc.

The effect of conjugation is again larger in the early growth stages of the floc up till about time step t=2000. In the later growth stages, resistant and nonresistant populations grow in more distinct protrusions at the edge of the floc but a much larger proportion of bacteria are nonresistant in this simulation than in the previous.

The population plot in the bottom right of Figure 3 reveals that resistant bacteria have a minor advantage and that their population increases faster than nonresistant when the overall population is small. As the size of the floc increases, the intersection where conjugation can occur between resistant and nonresistant bacteria becomes smaller compared to the intersection between bacteria and substrate rich media where most of the growth occurs. The resistant population is noticeably higher than the nonresistant at t=2000, demonstrating the contributing effect of conjugation. As the floc grows, conjugation loses its effectiveness and the nonresistant bacteria population eventually overtakes the population of the resistant bacteria. Moreover, it is observed that a longer recovery time also affects the relative population of transconjugants. In simulation 2 there are fewer transconjugants than simulation 1.

3.3 Effect of lower probability for competence

The third simulation is performed to examine the effect of the probability for competence on the spread of resistance. The probability for a bacterium being competent, $P_{competent}$, is reduced to 0.1 and the recovery time, *T*, is kept 200 time steps as in simulation 1. Apart from this, simulation 3 uses the same initial

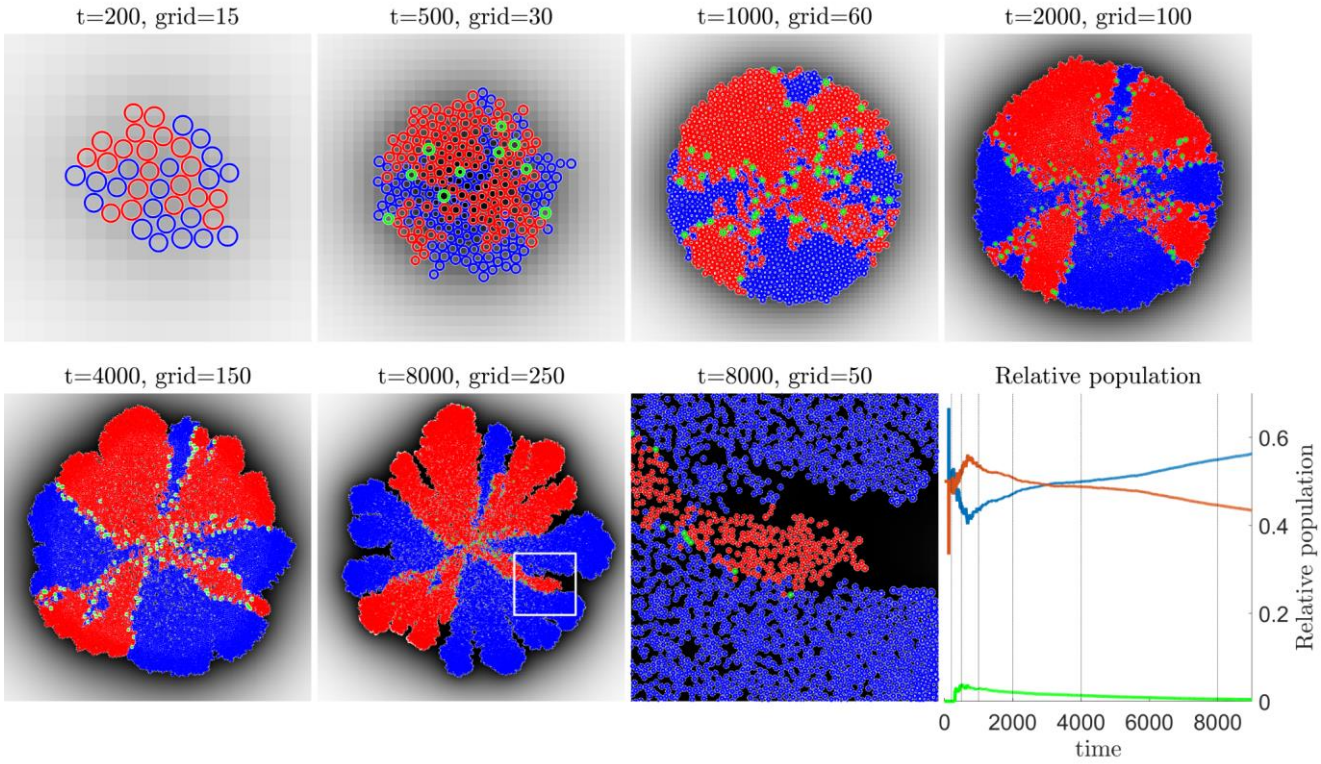

Figure 3. Population plot (lower right) and snapshots of floc development (other panels) from simulation 2. Timestep and size of the viewed grid are shown above the panels. Resistant (red), nonresistant bacteria (blue) and transconjugants (green). Substrate concentration is constant at the border of the nutrient grid and diffuses toward lower concentration, i.e., into the floc (grayscale, white is high concentration). Initially, at t=0 (not shown), ten resistant (red) and ten nonresistant bacteria are initialized in the middle of the environment. The probability of nonresistant bacteria being compatible is 20% while all resistant bacteria are competent. Conjugation recovery time is 300 time steps. At each time step, t, the simulation algorithm is run once.

conditions as in simulation 1, with ten resistant and ten nonresistant bacteria in the middle of the floc at t=0. The results of simulation 3 are shown in Figure 4, which shows the snapshot results and relative the population.

The overall growth and shape of the floc are similar to simulation 1, but the nonresistant bacteria population is higher. Initially, conjugation plays a significant role and transconjugants spread in the floc. However, in the later stage at t=2000, the floc starts to have a shape with clear distinct protrusions and the relative amount of transconjugants decrease.

The relative population plot in the bottom right of Figure 4 shows similarities with the results of simulation 1. It is observed that a lower value of the probability of competence has an impact on the relative population of resistant bacteria. It introduces a drop in the resistant bacteria population.

4 Discussion

The persistence of resistance and the interactions between bacteria with and without resistance are of utmost concern in a wastewater environment. In this work, relevant information about bacterial behaviour and interactions, especially on conjugation, are put together into an IbM. The model is a structurally realistic test environment for examining the effect of conjugation and nutrient limited growth on the spread of resistance in a bacterial floc. The model presented here is simplified but is still able to show that population size and substrate availability have notable effects in the floc.

A number of other mathematical models have been published to analyse bacterial interactions and improve our knowledge of the spread of antibiotic resistance (Birkegård *et al.*, 2018) and other biological phenomena. More specifically, IbMs have been used to shed light on interactions on the micro-level and to produce more mechanistically accurate representations of microbial systems (Kreft *et al.*, 2001; Hellweger et al., 2016). Moving aside from resistance spread, in particular, there have been attempts to make more general-purpose IbMs and solvers for bacterial systems, which in addition to basic functions like growth and substrate diffusion typically includes physical aspects like fluid flow, shear forces, and extracellular polymeric substance adhesion forces. Notable are iDynoMiCS (Lardon *et al.*, 2011) and NUFEB (Li *et al.*, 2019). Such IbMs may also include the possibility to have different

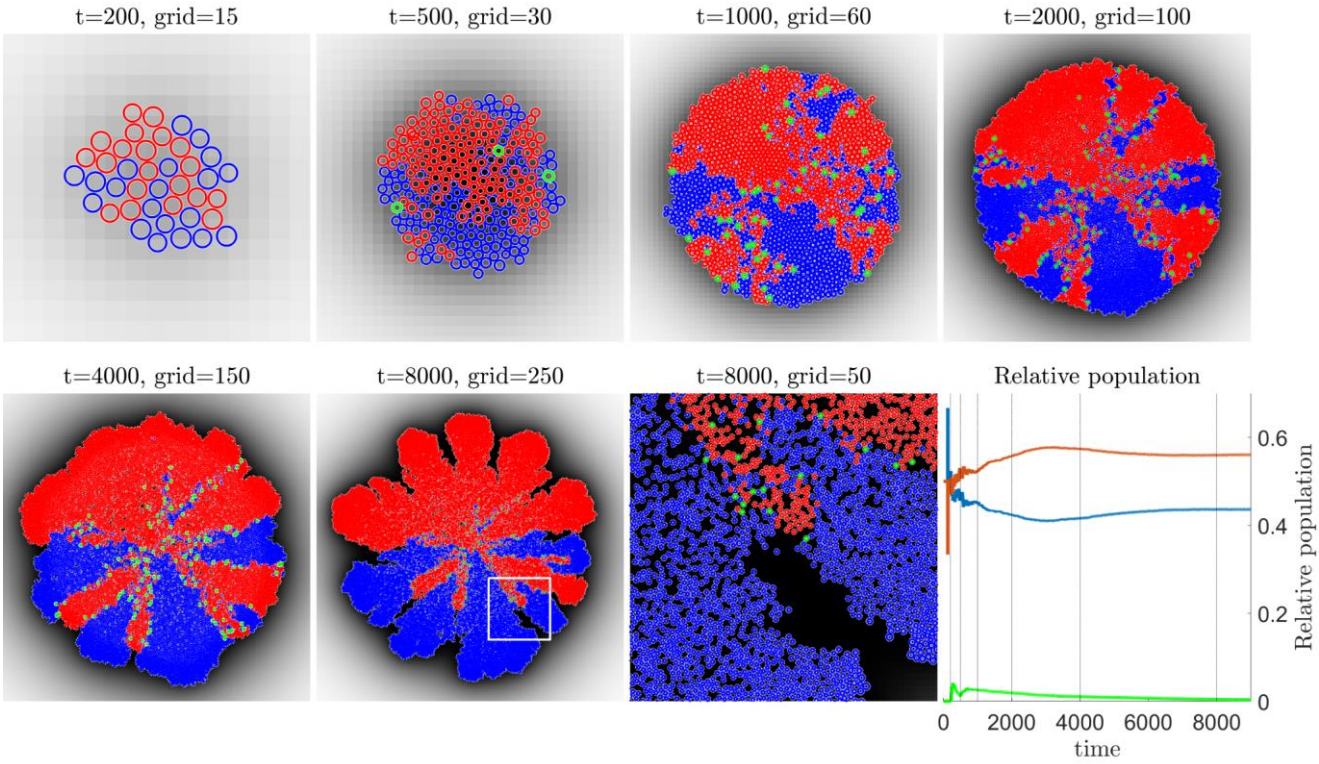

Figure 4. Population plot (lower right) and snapshots of floc development (other panels) from simulation 3. Timestep and size of the viewed grid are shown above the panels. Resistant (red), nonresistant bacteria (blue) and transconjugants (green). Substrate concentration is constant at the border of the nutrient grid and diffuses toward lower concentration, i.e., into the floc (grayscale, white is high concentration). Initially, at t=0 (not shown), ten resistant (red) and ten nonresistant bacteria are initialized in the middle of the environment. The probability of nonresistant bacteria being compatible is 10% while all resistant bacteria are competent. Conjugation recovery time is 200 time steps. At each time step, t, the simulation algorithm is run once.

species of bacteria in the system, e.g., species that are metabolically different like heterotrophic bacteria and autotrophic nitrifying bacteria. We will in the future examine the possibility to use such general-purpose bacterial IbMs for the task of modelling and simulating resistance spread, for example by including our model or parts of it into them, or by extending our model with functionality from them.

5 Conclusion and future development

In this paper, we have introduced an individual-based model for the spread of resistance in a bacterial floc through growth and horizontal gene transfer by conjugation. The attributes of each individual bacteria in the model includes metabolic processes such as substrate uptake and growth in addition to the processes of reproduction and conjugation. During the simulation, each bacterium in the model is updated according to an algorithm that considers the current state of the bacterium and its local neighbourhood. Simulations of the model show that the effect of conjugation varies as the floc grows. Conjugation can only occur between bacteria that are neighbours, and resistant and nonresistant bacteria seem to grow more and more on distinct protrusions as the floc and the overall population grows. Compared to population size, more conjugation occurs at the start of the simulation while it decreases in the later part of the simulation.

We plan to in the future work on finding ways to parameterize our current model with biological data from suitable experiments. And then to work on extending the model's functionality.

Acknowledgement

This work is supported by the European Union JPI-AMR grant Wastewater treatment plants as critical reservoirs for resistance genes (Gene-gas) through the Norwegian Research Council grant NFR 271177/H10.

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